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Review Article

Keratinocyte growth factor receptor tyrosine kinase antagonists for the prevention of metastatic cancer progression

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Abstract

The metastatic spread of cancer to secondary sites is a multistep process which has been correlated with highly motile behavior. It has been shown that the metastatic progression of cancer cells and enhanced invasive and motile behavior are regulated, in large part, by growth factors and cytokines. Often, stromal tissue surrounding the cancer cells produces growth factors which enhance cancer proliferation and progression to a metastatic phenotype. Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor (FGF) family. KGF is known to be associated with the proliferation, invasion and progression of many types of cancer. There is evidence that this growth factor may be an early signal in the progression of breast and other cancers. KGF binds to a specific tyrosine kinase receptor which mediates the KGF cellular response. Selective KGF receptor (KGFR) tyrosine kinase inhibitors may have promise for reducing the metastatic development of KGF-responsive cancers, and thus be therapeutically effective in reducing metastatic progression.

Introduction

The process of cancer metastasis involves a complex cascade which includes cell proliferation, release of proteolytic enzymes, invasion and establishment of a new microenvironment at the end organ (1, 2). However, one key feature of the metastatic process is cell motility. This is followed by penetration through the basal lamina and endothelial lining of capillaries, and the entry of tumor cells into the blood and lymph vascular systems, which permits widespread dissemination of highly motile cancer cells throughout the body. It appears that peptide growth factors are involved in metastatic progression and have a profound effect on cell motility (3).

Keratinocyte growth factor (KGF) was discovered in 1989 as part of an investigation to identify soluble factors in stromal tissue that enhance the growth of epithelial cells (4, 5). KGF is a 194-amino-acid protein and undergoes post-translational glycosylation which is not necessary for full biological activity (4). KGF, which was originally isolated from human embryonic lung fibroblasts (5), is a member of the fibroblast growth factor (FGF) family and has also been designated FGF-7 (6, 7). In addition to lung fibroblasts, KGF has also been identified in stromal tissue from human adult lung, skin, stomach, bladder, prostate and mammary tissue (4).

Although not produced by epithelial tissue, KGF from the surrounding stromal tissue stimulates DNA synthesis, proliferation and migration of epithelial cells (8, 9). These biological actions of KGF are thought to be involved in normal morphogenesis and tissue repair; however, KGF may contribute to tumor cell progression by enhancing cancer cell proliferation, motility and invasion (10-14). There is evidence that KGF and its receptor are associated with the metastatic progression of cancer (4, 15). Thus, KGF, the KGF receptor (KGFR) and related signal transduction pathways may represent promising therapeutic targets for preventing cancer metastasis. The involvement of KGF activity in cancer progression and the development of selective KGF signaling inhibitors as novel therapeutic agents will be discussed in this review.

KGF and KGFR signaling

KGFR (also known as FGFR2IIIb) is a splice variant of FGFR2 encoded by the *FGFR2* gene (16, 17). Thus, KGFR is a member of the FGF receptor (FGFR) family which are membrane-spanning tyrosine kinase (TK)

receptors consisting of four known peptides whose sequences are highly conserved (11).

It is well established that the target epithelial cells contain high-affinity KGFRs (6, 13, 17). *In situ* hybridization studies confirmed the specific mesenchymal distribution of KGF and epithelial distribution of KGFR in target tissue, and provided further evidence that KGF is a mesenchymally derived mediator of epithelial cell proliferation and migration (4, 18). It appears that KGF produced by fibroblasts and other stromal tissues acts in concert with hormones and other growth factors in the process of tissue morphogenesis and differentiation (9). For example, it has been demonstrated that KGF and KGFR are involved in the growth and branching morphogenesis of embryonic lung, mammary and prostate epithelium and other tissues (10, 19-21) .

KGF binding to KGFR is known to activate various signal transduction pathways, including extracellular signal-regulated kinases 1/2 (ERK1/2) and phosphatidylinositol 3-kinase (PI3K) (22-25). However, the signaling mechanism which mediates KGF-induced cancer cell motility appears to primarily involve the ERK1/2 pathway (25, 26). Accordingly, it has been reported that the ERK1/2 pathway mediates cell proliferation and motility associated with KGF actions in wound healing (27).

In a study employing cDNA microarray analysis of MCF7 breast cancer cells, it was found that KGF produced an increase in the mRNA levels of growth factor receptor-bound protein 2 (GRB2) and other ERK signaling intermediates (28). The *GRB2* gene is highly conserved among numerous species and the GRB2 protein is a ubiquitously expressed adapter protein; furthermore, GRB2 is known to transduce activated TK signaling to Ras (29), which could in turn activate the Raf/MEK/ERK1/2 pathway (30). It is known that downregulation of GRB2 protein expression by liposome-incorporated GRB2 antisense oligonucleotides can inhibit the proliferation of breast cancer cells in an ERK1/2-dependent manner (31). While GRB2-mediated signaling has not been specifically reported for KGFR, GRB2 is known to associate with other FGF receptors (32) and has been implicated in cell chemotaxis (33). Overexpression of GRB2 protein has been found in breast cancer cells and breast cancer tissue specimens (34, 35). KGF treatment doubled the expression of phospho-ERK1/2, while downregulation of GRB2 protein expression inhibited KGF-induced cell motility in human breast cancer cells (26). Moreover, ERK1/2 activity is known to be involved in the proliferation of endometrial carcinoma cells and in the invasion of stomach cancer cells induced by KGF (23, 25).

It has also been proposed that signal transduction via some FGFRs involves the additional binding of heparan sulfate, an abundant cell-surface molecule, to form a ternary signaling complex (36). Furthermore, the heparan proteoglycans on the cell surface may act to attract growth factors to the cell-surface receptors or serve as a reservoir for the growth factors in the vicinity of the cell membrane (37).

Involvement of KGF and KGFR in cancer

The metastatic dissemination of tumor cells to secondary sites has been correlated with highly motile behavior (38). It has been shown that the motile behavior of tumor cells is regulated, in part, by growth factors and cytokines (39, 40). In some instances, stromal tissue surrounding the tumor cells produces growth factors, which enhance tumor cell proliferation and progression to a metastatic phenotype (41, 42).

The mammary glands of adult female animals are remarkably sensitive to KGF (43). Systemic administration of KGF in adult male and female rats for 3-5 days was found to produce massive mammary ductal hyperplasia and an elevation of mitotic figures (43). Intraductal hyperplasia is known to be characteristic of premalignant breast lesions which lead to neoplasia. Similarly, Kitsberg and Leder (44) observed that female mice with a constitutively upregulated *KGF* transgene develop mammary epithelial hyperplasia, and all animals eventually developed metastatic mammary carcinomas. Consistent with this concept, a high level of KGF expression was observed in human primary breast tumor specimens (45). It has also been reported that KGF is a paracrine growth factor in breast cancer (46). However, highly malignant, metastatic breast cancer tissue expressed relatively little KGFR (47). It was observed that treatment with recombinant human KGF produced a profound stimulation of cell motility and an upregulation of the *KGFR* gene in estrogen receptor (ER)-positive human breast cancer cells, but this effect did not occur in ER-negative cell lines (48). The KGF motility response in breast cancer cells was shown to be concentration-dependent and characterized by an immediate increase in ruffling of the plasma membrane and cell scattering which continued for up to 48 h following KGF treatment (49). Changes in cell morphology associated with membrane ruffling and motility are believed to be associated with cytoskeletal re-organization and to be necessary for adhesion foci, cell-surface ligand-receptor binding and regulation of gene transduction (50, 51). Also, a significant alteration in the distribution of F-actin was observed in the cytoplasm of KGF-stimulated MCF7 cells (52). Furthermore, KGF and KGFR have been reported to enhance the progression of breast cancer by inhibiting normal apoptosis through the overexpression of Bcl-2 (53). Taken together, these observations suggest that KGF-mediated stimulation of breast epithelial proliferation and migration may be an early event in the molecular cascade which leads to breast cancer progression and metastasis (54).

In addition to breast cancer, KGF/KGFR signaling appears to be involved in the proliferation, invasion and malignancy of other estrogen- and androgen-dependent cancers, such as prostate (55-58), cervical (59), ovarian (60) and endometrial cancers (25, 61). The expression of KGF appears to be regulated by estradiol, progesterone, testosterone and gonadotropins (60). Furthermore, KGF expression correlates closely with ERα expression in human breast cancer tissue and the promoter region of

the *KGF* gene is known to contain a semi-palindromic sequence of the estrogen response element (62). In addition, other types of cancer, such as colorectal (63), lung (64) and stomach cancer (65), appear to be associated with KGF signaling. Therefore, KGF/KGFR signaling may be involved in the metastatic progression of many types of cancer.

Steele and co-workers (66) reported that KGFR is undetectable in normal ovarian epithelial cells, while 80% of the ovarian cancer samples expressed KGFR. It was suggested that the induction of KGFR expression is involved in ovarian cancer progression by making the premalignant tissue more sensitive to KGF stimulation (66, 67). Parrott and co-workers (60) reported that KGF produced in the tumor microenvironment may be involved in the progression of ovarian cancer. Similarly, KGFR was detected in carcinomas from 86% of cervical cancer patients, suggesting that it also mediates cervical cancer progression (59).

Both KGF and KGFR were found to be elevated in benign prostatic hypertrophy (BPH) and prostate cancer, where KGF appears to act as an autocrine factor in cancer progression (55, 56, 68). In clinical prostate cancer, the expression of KGF increases with progression and switches from stromal to epithelial cells, which permits stromal independence during metastatic progression (69). Furthermore, switching of the KGFR from the FGFR2IIIb (androgen-regulated receptor isoform) to the FGFR2IIIc isoform (which recognizes other FGF peptides) occurs during tumor progression and enhances unregulated tumor growth and invasion (41, 58, 70-72).

Similarly, KGF and KGFR signaling appears to be associated with the progression and both paracrine and autocrine stimulation of other nonendocrine cancers. Overexpression of KGF, KGFR, or both, has been reported in lung, colorectal, gastric and pancreatic carcinomas (63, 64, 73, 74). Interestingly, it was reported that KGF and KGFR expression levels were increased 5-fold within 28 days in a rat model of pancreatitis (75). Furthermore, KGF and KGFR overexpression and co-localization have been observed in human pancreatic cancer (73).

A cancer cDNA profiling array was used to examine the expression of KGFR from 154 tumor and paired normal samples representing 19 types of human cancer (76). The study demonstrated that KGFR is upregulated in endocrine and other types of cancer tissue (*i.e.,* uterus, cervix, vulva, prostate, testes and lung) at an early stage of cancer development. These results suggest that KGFR upregulation may be an early signal in the progression of these cancers and that KGFR expression levels may be a useful prognostic biomarker. On the other hand, it was found that KGFR was upregulated in more advanced tumors in other types of cancer (*i.e.,* ovarian, stomach, small intestine, rectal, bladder, trachea and pancreatic), while it was actually downregulated in some other types of cancer on the array (*i.e.,* skin, liver, colon and kidney). Thus, KGF signaling appears to be involved in the progression of many types of cancer.

Nonselective KGF antagonists

FGFs are a family of peptides which are known to bind to heparin. There is considerable evidence that heparin and cell-surface proteoglycans enhance the activity of some members of the FGF family (77). The heparan proteoglycans on the cell surface may act to attract growth factors to cell-surface receptors or may serve as a reservoir for the growth factors in the vicinity of the cell membrane (78). However, it has been shown that heparin inhibits the action of KGF, unlike other members of the FGF family (78, 79).

It has been known for decades that heparin reduces the growth of primary tumors and metastatic development in experimental models (77). Retrospective meta-analysis of clinical studies involving the use of heparin and/or low-molecular-weight heparin (LMWH) for the prevention of venous thromboembolism has demonstrated that the use of these agents is associated with a reduction in cancer-associated mortality (80). It is known that LMWH is more stable and has better pharmacokinetic properties than heparin. Accordingly, most of these studies found greater anticancer activity for LMWH (77, 81). The mechanism for this anticancer effect has not been clearly established. It has been suggested that inhibition of FGF effects on tumor angiogenesis could be responsible for the anticancer activity (77). However, heparin-mediated KGF inhibition may also play a role in this beneficial effect and the influence of KGF signaling on cancer progression supports this possibility.

A study on breast cancer cells compared the influence of heparin, LMWH and KGFR2β(IIIb)/Fc on KGF-induced cancer cell motility and proliferation. KGFR2β(IIIb)/Fc is a soluble chimera of an extracellular KGFR fragment (82). This KGFR fragment binds to KGF and is a much more selective KGF inhibitor than heparin-related compounds (83). This study demonstrated that heparin, LMWH and KGFR2β(IIIb)/Fc were equally effective KGF antagonists during the first several hours of treatment. However, after 2 h the heparin-mediated inhibition of KGF activity diminished, while LMWH and KGFR2β(IIIb)/Fc produced a more prolonged inhibitory effect (82).

It appears that these compounds have some potential for use in the treatment or prevention of cancer metastatic events associated with KGF. On the other hand, the anticoagulant activity and poor oral bioavailability of heparin-related compounds severely limit their chronic use in the treatment of cancer.

Selective KGFR TK antagonists

Deregulation of receptor TK activity and the related signal transduction pathways is known to be involved in the development of cancer and its progression (36, 84, 85). For example, it is well established that overexpression of the epidermal growth factor receptor (EGFR) is predictive of aggressive and metastatic tumors in breast and other cancers (86-88). Specific inhibitors of EGFR TK activity have been found to be effective therapeutically in the treatment of cancer (86, 89, 90).

Since it has been demonstrated that KGF enhances cancer progression, potent and selective KGFR TK antagonists may be highly effective therapeutic agents in the treatment or prevention of metastatic cancer progression.

It has been shown that there is a transition in the KGFR from the IIIb isoform in primary tumors, which is KGF-responsive, to the IIIc isoform in advanced cancer, which is unresponsive to KGF (58). This transition of the KGFR isoforms following cancer initiation suggests that KGFR activation is an early signal involved in the initiation of cell migration and progression to aggressive growth and metastasis. Thus, selective inhibition of KGFR (the FGFR2IIIb isoform) would provide an opportunity to prevent or reduce cancer progression to a malignant phenotype.

Development of small-molecule ATP-competitive inhibitors as selective KGFR TK inhibitors

KGF binds to KGFR, a member of the FGFR family of tyrosine kinase receptors consisting of four known peptides whose sequences are highly conserved (83). Since KGFR is a growth factor TK receptor, the design of small-molecule selective inhibitors of KGFR that compete with ATP for the catalytic site in the receptor is a viable approach (91, 92). The X-ray crystal structure of KGFR is not known; however, the X-ray crystal structure of FGFR1, another member of the FGFR family, has been determined. The FGFR1 TK domain has been crystallized and structures determined in complex with the inhibitors PD-173074 (protein data bank (PDB) file# 2FGI), SU-4984 (PDB # 1AGW) and SU-5402 (PDB # 1FGI), and this protein exhibited 86% homology with the KGFR TK domain (13, 17, 58).

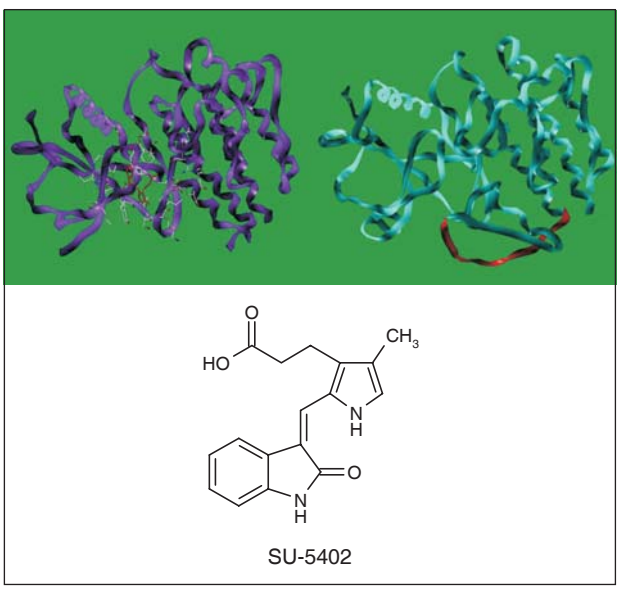

Fig 1. The crystal structure of FGFR1 (PBD ID: 1FGI) displayed with binding pocket amino acids (upper panel, left) and SU-5402. The KGFR homology model is displayed (upper panel, right) with the DINRVPEEQMTF stretch at the bottom of the model (in red).

A homology model of the KGFR TK domain using 2FGI as a template was constructed. This model was used to guide the design of novel ATP site-directed ligands. *In silico* site-directed mutagenesis was used to generate a model of the KGFR TK domain (93).

The crystal structure of the FGFR1 TK domain bound to the inhibitor PD-173074 served as the basis for a computed model using Sybyl. Amino acids Gly580 through Pro591 were missing from the structure and loop searching in the biopolymer module was used with *N*- and *C*-terminal anchor residues. A single loop from 1BVP was identified and the conformation was copied to the corresponding FGFR1 sequence. Mutation of the side-chains of FGFR1 to the corresponding side-chains of KGFR was performed, Tyr654 was phosphorylated and side-chains were minimized. A molecular dynamics simulation was performed at 300 K for 50,000 fs, with a Boltzmann distribution of initial velocities. Structures were averaged over time periods in which the atoms had obtained a stable trajectory. The averaged structures were minimized to a gradient of 0.5 kcal/mol/angstrom, followed by 100 steps of deepest descent minimization. The crystal structure of FGFR1 and the KGFR homology model are shown in Figure 1. This model has identified some key residues in the KGFR TK domain which differ from those found in FGFR1. A key difference in the TK domains of these proteins lies in a 12-amino-acid stretch near the entrance to the ATP binding site (Asn589 → Phe600 – DINRVPEEQMTF). Two residues in this segment, Asn586 and Pro587 of FGFR1, correspond to an aspartic acid and an isoleucine, respectively, in KGFR.

Virtual screening of combinatorial libraries was performed with FlexX within the Sybyl environment (Fig. 2). This flexible docking method used an incremental construction algorithm to place ligands into the active site. When performing docking of ligands in FGFR1, the bound ligands were used as a reference and the binding pocket was defined by amino acids in a 6.5 Å sphere from ligand atoms. The binding mode of SU-5402 in 1FGI was tested

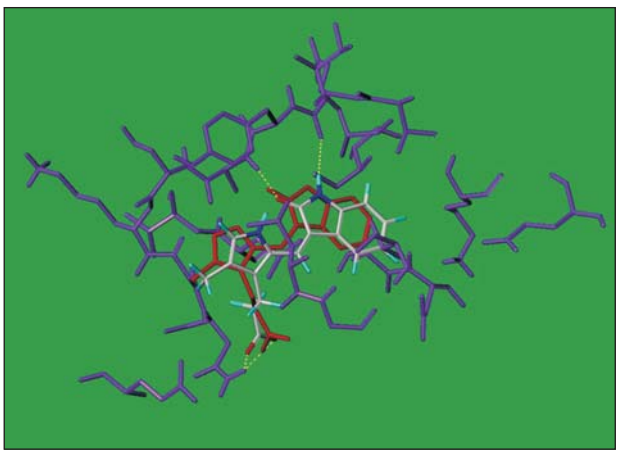

Fig 2. FlexX reproduces the binding mode of SU-5402 in 1FGI. Protein, reference ligand and docked SU-5402 (by atom type) are shown. Hydrogen bonds are dotted lines.

to validate the docking method. The ranking of the generated solutions was performed using a scoring function that estimates the free binding energy of the protein-ligand complex. The compounds with the highest estimated free binding energy contained indolinone, dihydroquinolinone, quinolinone and benzofuroquinolinone structural cores. A group of over 65 compounds with a high degree of predicted KGFR affinity was identified in this study (93).

Biological activity of the KGFR TK inhibitors

Three of the compounds identified with this modeling algorithm were synthesized and tested for their ability to inhibit KGF-mediated breast cancer cell proliferation and motility in a culture wounding model (93). It was observed that treatment of MCF7 cells with KGFR TK inhibitor compounds at a concentration of 20 μM reduced KGF-induced cell migration to approximately the same degree as the affinity of the compounds for the KGFR as predicted by the computer model. In addition, it was observed that the most potent KGF inhibitor produced a marked reduction in KGFR density on the cancer cells, which suggests that receptor targeting downregulates the expression of KGFR.

Conclusions

The metastatic spread of cancer cells is largely responsible for cancer mortality. Very few therapeutic modalities are available to selectively inhibit or reduce metastatic progression. Since KGF enhances the motility and proliferation of cancer cells, and since KGF signaling appears to be an early event associated with the progression of many types of cancer, potent and selective TK inhibitors hold promise as effective therapeutic agents. These studies indicate that the receptor modeling algorithm described in this review is capable of predicting highly effective and selective KGFR TK inhibitors which have the potential to be used therapeutically in the prevention of metastatic cancer progression.

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